INSTRUCTIONS

Micro BCA Protein Assay Kit

23235 0412.6

Number	Description
23235	**Micro BCA Protein Assay Kit**, sufficient reagents for 480 tube assays or 3200 microplate assays

Kit Contents:

Micro BCA Reagent A (MA), 240mL

Micro BCA Reagent B (MB), 240mL

Micro BCA Reagent C (MC), 12mL

Bovine Serum Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules containing bovine serum albumin (BSA) at 2.0mg/mL in a solution of 0.9% saline and 0.05% sodium azide

Storage: Upon receipt store product at room temperature. Product shipped at ambient temperature.

Note: If either Reagent MA or Reagent MB precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solutions. Discard any reagent that shows discoloration or evidence of microbial contamination.

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Introduction

The Thermo Scientific™ Micro BCA™ Protein Assay Kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. An adaptation of the Thermo Scientific™ BCA Protein Assay Kit (Product No. 23225), the Micro BCA Kit has been optimized for use with dilute protein samples (0.5-20µg/mL). The unique, patented method uses bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment.[1] A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations.

The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.[2] Studies with di-, tri- and tetrapeptides suggest that the extent of color formation is caused by more than the mere sum of individual color-producing functional groups.[2]

The Micro BCA Protein Assay Kit uses concentrated reagents and a protocol that utilizes an extended incubation time at an elevated temperature (60°C, Test Tube Procedure only). The result is an extremely sensitive colorimetric protein assay in a test tube or microplate assay format.

Note: For peptide sample concentration measurements, use the Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay or the Pierce™ Quantitative Colorimetric Peptide Assay (see Related Thermo Scientific Products).

Preparation of Standards and Working Reagent

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin (BSA) Standard ampule into several clean vials, preferably using a diluent that is similar to the sample buffer. Each 1mL ampule of 2.0mg/mL Albumin Standard is sufficient to prepare a set of diluted standards such that three replicates of each dilution may be included in the Test Tube Procedure.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	4.5mL	0.5mL of Stock	200µg/mL
B	8.0mL	2.0mL of vial A dilution	40µg/mL
C	4.0mL	4.0mL of vial B dilution	20µg/mL
D	4.0mL	4.0mL of vial C dilution	10µg/mL
E	4.0mL	4.0mL of vial D dilution	5µg/mL
F	4.0mL	4.0mL of vial E dilution	2.5µg/mL
G	4.8mL	3.2mL of vial F dilution	1µg/mL
H	4.0mL	4.0mL of vial G dilution	0.5µg/mL
I	8.0mL	0	0µg/mL = Blank

B. Preparation of the Micro BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

   Example: for the standard Test Tube Procedure with 3 unknowns and 2 replicates of each sample:

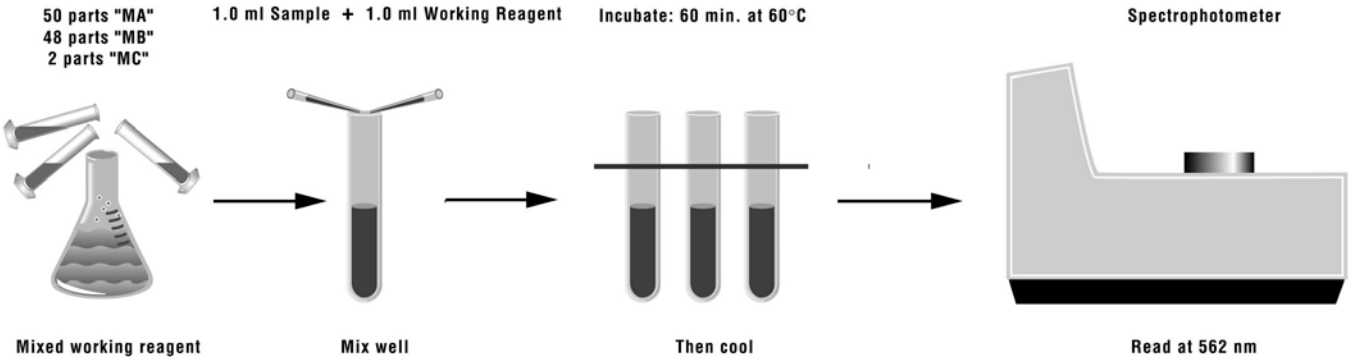
   (9 standards + 3 unknowns) × (2 replicates) × (1mL) = 24mL WR required (round up to 25mL)

   Note: 1mL of the WR is required for each sample in the Test Tube Procedure, while only 150µL of WR is required for each sample in the Microplate Procedure.

2. Prepare WR by mixing 25 parts of Micro BCA Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC). For the above example, combine 12.5mL of Reagent MA and 12.0mL Reagent MB with 0.5mL of Reagent MC.

   Note: When Reagent MC is initially added to Reagents MA and MB, turbidity occurs that quickly disappears upon mixing to yield a clear-green solution. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for one day when stored in a closed container at room temperature (RT). It is not necessary to protect the solution from light.

Procedure Summary (Test Tube Procedure)



Test Tube Procedure (linear working range of 0.5-20µg/mL)

1. Pipette 1.0mL of each standard and unknown sample replicate into appropriately labeled test tubes.
2. Add 1.0mL of the WR to each tube and mix well.
3. Cover tubes and incubate at 60°C in a water bath for 1 hour.
4. Cool all tubes to room temperature (RT).
5. With the spectrophotometer set to 562nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

   Note: Color development continues even after cooling to RT. However, the rate of development at RT is sufficient low that no significant error is introduced if all absorbance measurements are made within a 10-minute period.
6. Subtract the average 562nm absorbance reading of the Blank standard replicates from the 562nm reading of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562nm reading for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (linear working range of 2-40µg/mL)

1. Pipette 150µL of each standard or unknown sample replicate into a microplate well (Product No. 15041**)**.
2. Add 150µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate using Sealing Tape for 96-Well Plates (Product No. 15036) and incubate at 37°C for 2 hours.

   Note: Limit incubations of microplate to less than or equal to 37°C, otherwise high background and aberrant color development may result. Most polystyrene assay plates deform, leach, and become cloudy at 60°C.
4. Cool plate to room temperature (RT).
5. Measure the absorbance at or near 562nm on a plate reader.
6. Subtract the average 562nm absorbance reading of the Blank standard replicates from the 562nm reading of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562nm reading for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: If using curve-fitting software, use a best-fit polynomial equation rather than a linear equation for the standard curve. If plotting results by hand, a point-to-point fit is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze, desalt, or dilute sample Increase copper concentration in working reagent (e.g., use more Reagent MC)
Blank absorbance is OK, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze, desalt, or dilute sample
	Color measured at the wrong wavelength	Measure the absorbance at 562nm
Color of samples appears darker than expected	Protein concentration is too high	Dilute sample
	Sample contains lipids or lipoproteins	Add 2% SDS to the sample[3]
All tubes (including blank) are dark purple	Buffer contains a reducing agent	Dialyze or dilute sample
	Buffer contains a thiol	
	Buffer contains biogenic amines (catecholamines)	
Need to measure color at a different wavelength	Spectrophotometer or plate reader does not have 562nm filter	Wavelengths between 540nm and 590nm can be used, but standard curve slope and overall assay sensitivity will be decreased. See Tech Tip on website

Additional Information

A. Interfering Substances

Certain substances are known to interfere with the Micro BCA Assay including those with reducing potential, chelating agents, and strong acids or bases. Avoid the following substances as components of the sample buffer:

Ascorbic Acid	Hydrogen Peroxide	Iron	Reducing Sugars
Catecholamines	Hydrazides	Lipids	Tryptophan
Cysteine	Impure Glycerol	Phenol Red	Tyrosine
EGTA	Impure Sucrose	Reducing Agents	Uric Acid

Maximum compatible concentrations for many substances in the Test Tube Procedure are listed in Table 2 (see last page). Substances were considered compatible at the indicated concentration if the error in protein concentration estimation caused by the presence of the substance in the sample was less than or equal to 10%. The substances were tested using WR prepared immediately before each experiment. The Blank-corrected 562nm absorbance measurements (for the 10µg/mL BSA standard + substance) were compared to the net 562nm readings of the same standard prepared in 0.9% saline.

B. Strategies for Eliminating or Minimizing the Effects of Interfering Substances

The effects of interfering substances in the Micro BCA Protein Assay may be eliminated or overcome by several methods.

- Remove the interfering substance by dialysis or gel filtration (see Related Thermo Scientific Products).
- Dilute the sample until the substance no longer interferes. (This is only effective for relatively concentrated samples.)
- Precipitate proteins with acetone or trichloroacetic acid (TCA).[4]
- Increase the amount of copper in the WR (prepare WR using a greater proportion of Reagent MC; e.g., MA:MB:MC equal to 25:24:2 or 25:24:3), which may eliminate interference by copper chelating agents.
  Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

C. Response Characteristics for Different Proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins (Table 3). These differences relate to amino acid sequence, pI, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. Most protein assay methods use BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined. Pierce Albumin Standard (BSA) (Product No. 23209) provides a consistent standard for protein estimations. Nevertheless, individual proteins, including BSA and IgG, differ slightly in their color responses in the Micro BCA Assay (Figure 1). Therefore, for maximum accuracy use a purified (known concentration) sample of the target protein as the assay standard.

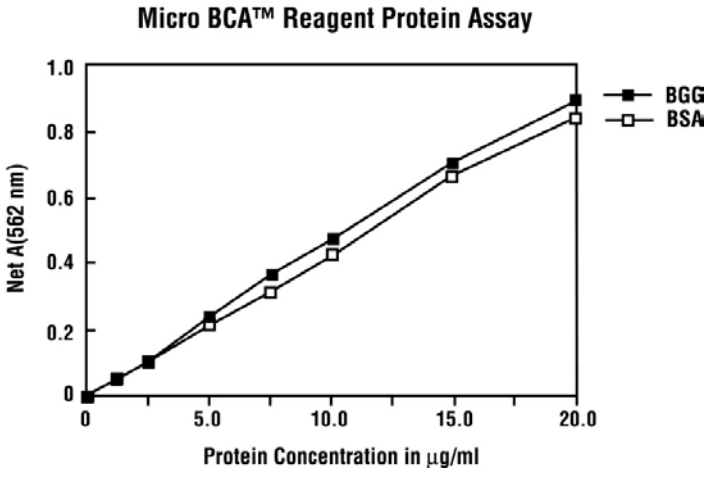


Figure 1. Typical color response curves for BSA and BGG using the Test Tube Procedure.

Table 3. Protein-to-Protein Variation. Absorbance ratios (562nm) for proteins relative to BSA using the Test Tube Procedure.

Ratio = (avg. "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.80
α-Chymotrypsinogen, bovine	0.99
Cytochrome C, horse heart	1.11
Gamma globulin, bovine	0.95
IgG, bovine	1.12
IgG, human	1.03
IgG, mouse	1.23
IgG, rabbit	1.12
IgG, sheep	1.14
Insulin, bovine pancreas	1.22
Myoglobin, horse heart	0.92
Ovalbumin	1.08
Transferrin, human	0.98
Average Ratio	**1.05**
Standard Deviation	**0.12**
Coefficient of Variation	**11.4%**

D. Alternative Total Protein Assay Reagents

If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, try the Thermo Scientific™ Coomassie Plus™ **(**Bradford) Protein Assay Kit (Product No. 23236), which is less sensitive to such substances.

E. Cleaning and Re-using Glassware

Care must be exercised when re-using glassware. The Micro BCA WR is sensitive to metal ions, especially copper ions. All glassware must be cleaned and then given a thorough final rinse with ultrapure water.

Related Thermo Scientific Products

15041	**Pierce™ 96-Well Plates,** 100/pkg
15075	**Reagent Reservoirs,** 50mL, 200/pkg
15036	**Sealing Tape for 96-Well Plates,** 100/pkg
23209	**Bovine Serum Albumin (BSA) Standard Ampules, 2mg/mL**, 10 × 1mL
23212	**Bovine Gamma Globulin (BGG) Standard Ampules, 2mg/mL**, 10 × 1mL
23290	**Pierce Quantitative Fluorometric Peptide Assay**
23275	**Pierce Quantitative Colorimetric Peptide Assay**
23236	**Coomassie Plus™ (Bradford) Protein Assay Kit**
89882	**Zeba™ Spin Desalting Columns, 0.5mL**
89889	**Zeba Spin Desalting Columns, 2mL**
69576	**Slide-A-Lyzer™ MINI Dialysis Units**
69576	**Slide-A-Lyzer Dialysis Cassettes**

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Table 2. Compatible Substance Concentrations in the Micro BCA Protein Assay (see text for details).

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	10mM
Ammonium sulfate	--------
Bicine, pH 8.4	2mM
Bis-Tris, pH 6.5	0.2mM
Borate (50mM), pH 8.5 (#28384)	1:4 dilution*
B-PER® Reagent (#78248)	1:10 dilution*
Calcium chloride in TBS, pH 7.2	10mM
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (#28382)	undiluted
Cesium bicarbonate	100mM
CHES, pH 9.0	100mM
Na-Citrate (0.6 M), Na-Carbonate (0.1 M), pH 9.0 (#28388)	1:600 dilution*
Na-Citrate (0.6 M), MOPS (0.1 M), pH 7.5 (#28386)	1:600 dilution*
Cobalt chloride in TBS, pH 7.2	--------
EPPS, pH 8.0	100mM
Ferric chloride in TBS, pH 7.2	0.5mM
Glycine	n/a
Guanidine•HCl	4M
HEPES, pH 7.5	100mM
Imidazole, pH 7.0	12.5mM
MES, pH 6.1	100mM
MES (0.1M), NaCl (0.9%), pH 4.7 (#28390)	1:4 dilution*
MOPS, pH 7.2	100mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	0.2mM
PBS; Phosphate (0.1M), NaCl (0.15M), pH 7.2 (#28372)	undiluted
PIPES, pH 6.8	100mM
RIPA lysis buffer; 50mM Tris, 150mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	1:10 dilution*
Sodium acetate, pH 4.8	200mM
Sodium azide	0.2%
Sodium bicarbonate	100mM
Sodium chloride	1M
Sodium citrate, pH 4.8 (or pH 6.4)	5mM (16.7mM)
Sodium phosphate	100mM
Tricine, pH 8.0	2.5mM
Triethanolamine, pH 7.8	0.5mM
Tris	50mM
TBS; Tris (25mM), NaCl (0.15M), pH 7.6 (#28376)	1:10 dilution*
Tris (25mM), Glycine (192mM), pH 8.0 (#28380)	1:10 dilution*
Tris (25mM), Glycine (192mM), SDS (0.1%), pH 8.3 (#28378)	undiluted
Zinc chloride in TBS, pH 7.2	0.5mM

Substance	Compatible Concentration
Detergents**	
Brij™-35	5.0%
Brij-56, Brij-58	1.0%
CHAPS (CHAPSO)	1.0% (5.0%)
Deoxycholic acid	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl β-glucoside	0.1%
Octyl β-thioglucopyranoside	5.0%
SDS	5.0%
Span™ 20	1.0%
Triton™ X-100	5.0%
Triton X-114	0.05%
Triton X-305, X-405	1.0%
Tween™-20, Tween-80	5.0%
Tween-60	0.5%
Zwittergent™ 3-14	--------
Chelating agents	
EDTA	0.5mM
EGTA	--------
Sodium citrate, pH 4.8 (or pH 6.4)	5mM (16.7mM)
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	--------
Ascorbic acid	--------
Cysteine	--------
Dithioerythritol (DTE)	--------
Dithiothreitol (DTT)	--------
Glucose	1mM
2-Mercaptoethanol	1mM
Thimerosal	--------
Misc. Reagents & Solvents	
Acetone	1.0%
Acetonitrile	1.0%
Aprotinin	1mg/L
DMF, DMSO	1.0%
Ethanol	1.0%
Glycerol (Fresh)	1.0%
Hydrazide	--------
Hydrides (Na_2BH_4 or $NaCNBH_3$)	--------
Hydrochloric Acid	10mM
Leupeptin	10mg/L
Methanol	1.0%
Phenol Red	--------
PMSF	1mM
Sodium Hydroxide	50mM
Sucrose	4%
TLCK	0.1mg/L
TPCK	0.1mg/L
Urea	3M
o-Vanadate (sodium salt), in PBS, pH 7.2	1mM

* Diluted with ultrapure water
** Detergents were tested using high-purity Thermo Scientific™ Surfact-Amps™ Products, which have low peroxide content
--- Dashed-line entry indicates that the material is incompatible with the assay

How to use a protein assay standard curve

TR0057.4

Introduction

Typical protein assays are used to determine protein concentration by comparing the assay response of a sample to that of a standard whose concentration is known. Protein samples and protein standards are processed in the same manner by mixing them with assay reagent and using a spectrophotometer to measure the absorbances. While protein quantitation from absorbance values is straightforward, one common source of confusion is the assumption that dilution of the sample with assay reagent is a necessary consideration. This Tech Tip describes how to properly calculate sample protein concentrations using a standard curve. Thermo Scientific® Pierce Protein Assays are used as examples, but the principles apply to protein assay methods in general.

Fundamental Principles of Standard Curve Assays

A. Identically assayed samples are directly comparable ("Equal treatment means equal opportunity.")

Sample assay responses are directly comparable to each other if they are processed in exactly the same manner. Variance in protein quantity is the only possible cause for differences in final absorbance (color intensity) if samples are dissolved in the same buffer and the same stock solution of assay reagent is used for all samples. Of course, different proteins will generate different absorbance values even at the same concentration, an issue that is discussed more fully in Pierce® Protein Assay product instructions (see Related Products).

B. Units in equals units out ("What you put in is what you get out.")

The unit of measure used to express the standards is by definition the same unit of measure associated with the calculated value for the unknown sample (i.e., final results for unknown samples will be expressed in the same unit of measure as was used for the standards). For example, if the standards are expressed as micrograms per milliliter, then the value for the unknown sample, which is determined by comparison to the standard curve, is also expressed as micrograms per milliliter.

The Total Amount of Protein Per Well is Irrelevant

With these principles of standard curve assays in mind, one can easily understand why it is neither necessary nor even helpful to know the actual amount (e.g., micrograms) of protein applied to each well or cuvette of the assay. Consider a simple example in which the Coomassie Plus Protein Assay Kit (Product No. 23236) is used to assay two protein samples: a test sample whose concentration is not known, and a standard whose concentration is 1mg/mL (= 1000µg/mL). (Ordinarily, an entire set of standards is necessary to establish a response curve, but this is a simplified example.)

In the microplate protocol, one adds 10µL of sample (test or standard) and 300µL of assay reagent per well. Because 10µL of the standard sample is added to a well, there is 0.010mL × 1000µg/mL = 10µg of protein in the well. If the assay results in the test sample having the same final absorbance as the standard sample, then the conclusion is that the test sample contains the same amount of protein as the standard sample. Because there was 10µg of standard/well, one could report the determined concentration of test sample as "10µg/well." However, the amount of protein per well is almost certainly not the value of interest; instead, one usually wants to know the protein concentration of the original test sample. Because the original standard was 1000µg/mL, the test sample that produced the same absorbance in the assay also must be 1000µg/mL.

The Protein Concentration in Assay Reagent is Irrelevant

Furthermore, it is neither necessary nor helpful to know the protein concentration as it exists when diluted in assay reagent. In the above example, because the 10µg standard was diluted to 310µL after adding of 300µL of assay reagent, the final concentration in the well is 10µg/310µL = 0.0323µg/µL = 32.3µg/mL. Therefore, one could report the determined concentration of test sample as "32.3µg/mL." However, the protein concentration when diluted by assay reagent is almost certainly not the value of interest; instead, one wants to know the protein concentration of the original test sample. Because the original standard was 1000µg/mL, the test sample that produced the same assay absorbance also must be 1000µg/mL.

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How to Apply Dilution Factors

One situation in which the dilution factor is important to consider is when the original sample has been pre-diluted relative to the standard sample. Continuing with the same example, suppose that the original protein sample is actually known to be approximately 5mg/mL. This is too concentrated to be assayed by the Coomassie Plus Protein Assay Kit, whose assay range in the standard microplate protocol is 100-1500µg/mL. However, one could dilute it 5-fold in buffer (i.e., 1 part sample plus 4 parts buffer) and then use that diluted sample as the test sample in the protein assay. If the test sample produces the same absorbance as the 1000µg/mL standard sample, then one can conclude that the test (5-fold diluted) sample is 1000µg/mL, and therefore the original (undiluted) sample is 5 × 1000µg/mL = 5000 µg/mL = 5mg/mL.

How to Interpolate on a Complete Standard Curve

Unlike the example described above, most assays use an entire set of protein standards whose concentrations span the effective assay range (e.g., 100-1500µg/mL for the Coomassie Plus Protein Assay Kit). Rarely, if ever, will the test sample produce an assay response that corresponds exactly to one of the specific standard samples. Therefore, a method is needed to calculate or interpolate between the Standard sample points. Most modern plate readers and spectrophotometers have associated software that automatically plots a linear or curvilinear regression line through the standard points, interpolates the test samples on that regression line, and reports the calculated value.

Consider the following example involving a set of six standard points (0, 250, 500, 1000, 1500 and 2000µg/mL). The same point-to-point relationship for this set of standards is plotted in Figures 1 and 2, but different methods were used to determine a mathematical equation that describes this absorbance-concentration relationship. If a test sample results in an absorbance of 0.6, then one must interpolate between values obtained for the 500 and 1000µg/mL standards to determine the test sample concentration.

On a merely graphical basis, one can see that the test sample must be ~ 650µg/mL.

- The line segment AB in the point-to-point graph is described by the equation y = 0.0006(x + 333.33).
- Solving this equation for x gives x = 1666.7y - 333.33.
- If y = 0.6, then x = 667µg/mL.

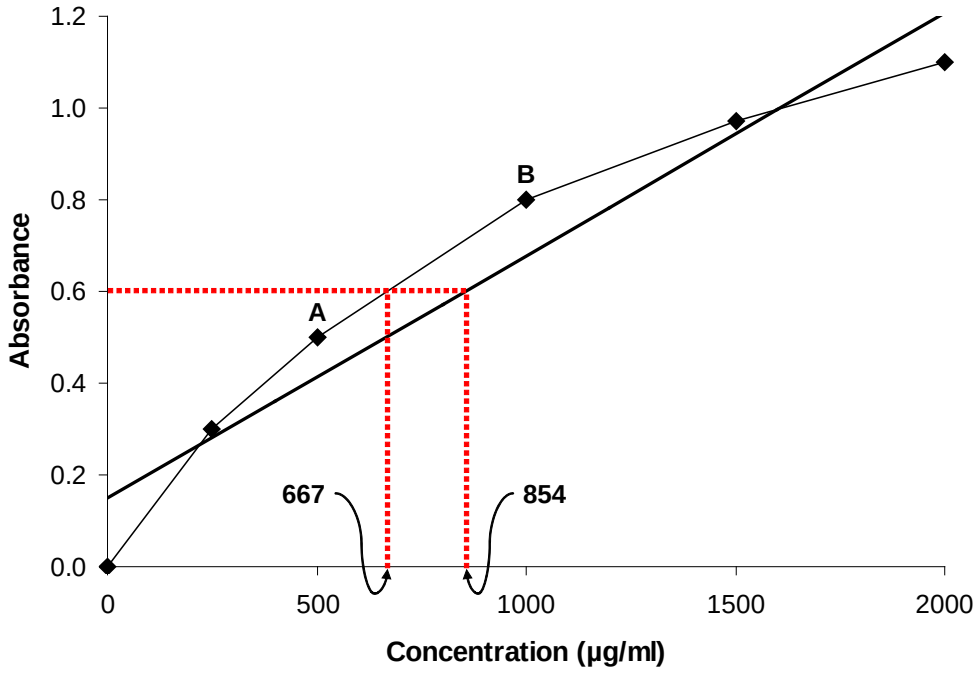

Figure 1. Example standard curve involving six points. The thin line is a point-to-point graph through the plotted standards. The thick line is linear regression for the entire set of standard points. Dashed lines represent interpolations for a test sample having absorbance 0.6. See text for additional details.

Many researchers plot a linear regression for the entire set of standards, assuming that the overall relationship between concentration and absorbance is best described by a straight line. The thick, straight line in Figure 1 is the linear regression that best describes the entire set of standard points ($R^2 = 0.9355$).

- The equation for this line is y = 0.00056x + 0.1174.
- Solving for x gives x = 1770.4y - 207.91.
- For y = 0.6, x = 854µg/mL.

As is obvious from the graph, this linear regression does not provide a good basis for interpolating test sample concentrations relative to assay results for the standards.

The best method for interpolation in this example is by reference to a curvilinear regression, in this case a 3-parameter polynomial equation that can be calculated by most plate reader software or standard spreadsheet programs (see box below).

- When solved for x, the 3-parameter equation ($R^2 = 0.9997$) is $x = 1372.2y^3 - 769.01y^2 + 1004.2y - 2.9373$.
- For y = 0.6, x = 619µg/mL.

On a merely graphical basis (Figure 2), this method can be seen as the most accurate for interpolating the test sample. If one had included a 750µg/mL standard, it surely would have occurred on this 3-parameter trend line rather than on the straight line segment AB or on the linear regression displayed in Figure 1.

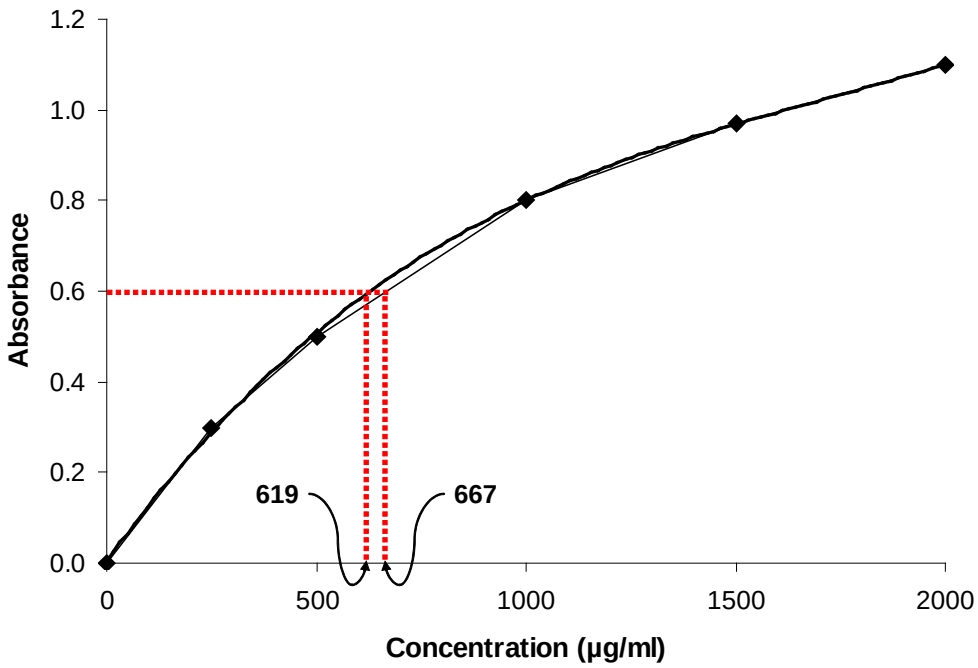

Figure 2. Example standard curve involving six points. The thin line is a point-to-point graph through the plotted standards. The thick line is a 3-parameter regression for the entire set of standard points. Dashed lines represent interpolations for a test sample having absorbance 0.6. See text for additional details.

Using Microsoft Excel to plot and apply standard curve

A protein assay, such the BCA Protein Assay, is an excellent tool for estimating the protein concentration of a sample. The intensity of the colored reaction product is a direct function of protein amount that can be determined by comparing its absorbance value to a standard curve. Plotting a graph with the absorbance value as the dependent variable (Y-axis) and concentration as the independent variable (X-axis), results in an equation formatted as follows: $y = ax^2 + bx + c$, where solving for x determines the protein concentration of the sample. By switching the axes and plotting protein concentration as the dependent variable on the Y-axis and absorbance as the independent variable on the X-axis, the protein concentration is represented by y and the equation is much easier to solve.

To use Excel for generating such an equation, enter the concentration values for the standards in Column A and their corresponding absorbance data in Column B. Highlight both columns and from the Insert menu select Chart and XY (Scatter). Click on the resulting graph and select Add Trendline from the Chart menu. While viewing the graph next to the open Format Trendline window, choose Polynomial and set the Order to 2, 3 or 4 until the best-fit appears. Check the box near the bottom called Display Equation on Chart; then close the Format Trendline window. Use the resulting equation to determine protein concentration (y) of an unknown sample by inserting the sample's absorbance value (x).

A Note About Interfering Substances

This Tech Tip has focused on proper calculation of test sample concentrations relative to the standard samples. The possible effects of interfering substances were not discussed because the assumption was that all protein samples were treated exactly the same, including the buffers in which the proteins were dissolved. In this situation, any interference caused by components of the buffers is exactly the same for both test and standard samples.

Nevertheless, interference by nonprotein substances in the samples that block or contribute to the assay color reaction is an important issue for any protein assay system. The instructions for the different Pierce Protein Assay Kits (see Related Products) describe the classes of compounds that are known to interfere, as well as several techniques for eliminating or minimizing their effects. The Reducing Agent Compatible BCA Protein Assay Kits (Product No. 23250 and 23252) contain a treatment reagent for specific eliminating interference by dithiothrietol (DTT) and other reducing agents that are commonly present in cell lysate and other protein samples.

Related Thermo Scientific Pierce Products

23209	**Bovine Serum Albumin Standard Ampules, 2 mg/ml**, 10 × 1mL ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide
23208	**Bovine Serum Albumin Standard Pre-Diluted Set**, 7 × 3.5mL aliquots in the range of 125-2000 µg/ml
23212	**Bovine Gamma Globulin Standard Ampules, 2 mg/ml**, 10 x 1mL ampules
23213	**Bovine Gamma Globulin Standard Pre-Diluted Set**, 7 × 3.5mL aliquots in the range of 125-2000 µg/mL
23225	**BCA Protein Assay Kit**, working range 20-2000µg/mL
23235	**Micro BCA Protein Assay Kit**, working range of 0.5-20µg/mL
23250	**BCA Protein Assay Kit – Reducing Agent Compatible**, working range 125-2000µg/mL
23250	**Microplate BCA Protein Assay Kit – Reducing Agent Compatible**
23236	**Coomassie Plus (Bradford) Assay Kit**, working range of 1-1500µg/mL
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**, sufficient reagents to pre-treat 500 samples to remove interfering substances before total protein quantitation

Current versions of product instructions are available at *www.thermoscientific.com/pierce*. Call 800-874-3723 or contact your local distributor.

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TECH TIP #8

Eliminate interfering substances from samples for BCA protein assays

TR0008.3

Introduction

Thermo Scientific® Pierce BCA Protein Assays are one of the great workhorses for measuring the protein concentration of a sample. One of the best features of the Pierce® BCA Protein Assay method is its compatibility with many detergents at concentrations up to 1 or 5% (commonly used concentrations for solubilizing proteins from cell membranes). The upper limits of compatibility for a wide variety of substances are listed in the instructions for the BCA Protein Assay Kit (Product No. 23225 or 23227). Some substances, including most reducing agents, interfere at even low concentrations in the BCA Assay. Examples include 5 mM DTT (for the BCA and Micro BCA™ Assays) and 0.1 M Glycine (for the Micro BCA Assay). Interference by individual substances can often be overcome by simple means, but some sample buffers contain several components that interact to produce an interference that is not so easily identified and eliminated.

Several strategies exist for overcoming or eliminating sample incompatibility with protein assays. The simplest method is to assay the sample after diluting it several-fold in a compatible buffer. If the starting concentration of protein is sufficient to remain within the protein assay working range upon its dilution, then this method will often reduce the amount of interfering substance in the sample to the point where it no longer interferes. Another method is to dialyze or desalt samples into a buffer that is compatible with the assay.

When disulfide reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) are the sole cause of the incompatibility, reagents can be used to chemically block the interfering sulfhydryl groups. The Reducing Agent Compatible BCA Protein Assay Kits (Product No. 23250 and 23252) include an optimized blocking reagent and protocol for samples containing reducing agents.

Finally, precipitation can be used to eliminate interfering substances, a general method for which is given in this Tech Tip. After causing the protein to precipitate with acetone or trichloroacetic acid (TCA), the supernatant containing the interfering substance can be removed. Then the protein pellet is dissolved in the assay working reagent, and the protein assayed performed as usual. Because the assay standards are processed in parallel with the protein samples, the rate of protein recovery caused by the precipitation process is automatically accounted for in the standard curve. Alternatively, use the Thermo Scientific Compat-Able™ Protein Assay Preparation Reagent Set (Product No. 23215, see Related Products), which includes optimized reagents and protocol for performing such precipitations.

Important Notes:

- The choice between acetone and TCA methods depends on which reagent is available in the lab and the properties of the protein and buffer sample being processed. Empirical testing will be necessary to establish which method is most effective for a given protein, buffer and incompatible substance.
- An assumption in these procedures is that the standard protein precipitates and redissolves with similar efficiency to the test protein sample. This assumption may not be valid in all circumstances and underscores the importance of using a protein standard that is as similar as possible in properties to the test protein.
- Single and double precipitation protocols may result in less linear standard curves than non-precipitated standards. Therefore, avoid using these precipitation methods with the Micro BCA Protein Assay if it is necessary to detect slight variations in protein concentrations (e.g.,18 µg/ml vs. 20 µg/ml), especially at the lower end of detection range. Larger differences in protein concentrations (e.g., 15 µg/ml vs. 20 µg/ml) should be detectable after a single precipitation.
- The procedures presented here are written in volumes sufficient to perform BCA or Micro BCA Protein Assays using the Test Tube Protocols. Obviously, an aliquot of the dissolved protein pellet resulting from these procedures could be transferred to microplate wells for absorbance measurement in that format. It may be possible to scale down the entire precipitation methods for direct use in the Microplate Protocols, but this has not been tested. Also, it may be possible to concentrate a dilute large-volume sample by scaling up the precipitation step while maintaining or reducing the volume of working reagent used to dissolve and assay the pellet.

TCA Precipitation Procedure

A. Materials Required

- Protein Standards: prepare according to the instructions for the BCA Protein Assay Reagent Kit (Product No. 23225)
- TCA reagent: 72% (w/v) trichloroacetic acid in ultrapure water
- Sodium deoxycholate reagent: 0.15% (w/v) sodium deoxycholate in ultrapure water
- BCA Working Reagent: (50 parts Reagent A + 1 part Reagent B), prepare according to the instructions for the BCA Protein Assay Reagent Kit (Product No. 23225, 23227)
- SDS reagent: 5% SDS (w/v) in 0.1 N sodium hydroxide (NaOH)
- Microcentrifuge tubes and benchtop microcentrifuge

B. Protocol for BCA Protein Assay (Product No. 23225, 23227)

1. Pipette 50 µl of each protein standard (including a blank) and sample into microcentrifuge tubes in triplicate.
2. Add 450 µl of ultrapure water.
3. Add 100 µl of the sodium deoxycholate reagent.
4. Add 100 µl of the TCA reagent and incubate 10 minutes at room temperature (RT).
5. Vortex, then centrifuge 10 minutes in a microcentrifuge at maximum speed.
6. Remove the supernatant, being careful not to disturb the pellet.

   Note: If two precipitations are necessary to remove the interfering substance, dissolve the pellet in 100 µl ultrapure water and then repeat steps 3-6 one time.
7. Add 50 µl of SDS reagent to completely dissolve the protein pellet.

   Note: As used in this protocol, 5% SDS is compatible with the BCA Protein Assay.
8. Add 1 ml of BCA Working Reagent.
9. Vortex, then incubate for 30 minutes at 37° C.
10. Measure the absorbance at 562 nm.
11. Plot a standard curve based on absorbance of the protein standards and determine the protein concentration of each unknown sample according to the BCA Protein Assay Kit instructions.

Acetone Precipitation Procedures

A. Materials Required

- Protein Standards: prepare according to the instructions for the BCA Protein Assay Reagent Kit (Product No. 23225)
- Cold acetone, -20°C
- BCA or Micro BCA Working Reagent: prepare according to the product instructions for the BCA Protein Assay Reagent Kit (Product No. 23225, 23227) or Micro BCA Protein Assay Reagent Kit (Product No. 23235)
- Microcentrifuge tubes and benchtop microcentrifuge

B. Protocol for BCA Protein Assay

1. Pipette 50 µl of each protein standard (including a blank) and sample into 1.5 ml microcentrifuge tubes in triplicate.
2. Add 200 µl of cold (-20°C) acetone to each tube.
3. Vortex and incubate 30 minutes at -20°C.
4. Centrifuge 10 minutes at maximum speed in a microcentrifuge.
5. Pour off the supernatants and allow the acetone to evaporate from the tubes at room temperature (RT) for 30 minutes.

   Note: If two precipitations are necessary to remove the interfering substance, repeat Steps 2-5 before proceeding.

6. Add 50 µl of ultrapure water to the protein pellets and vortex.
7. Add 1 ml of BCA Working Solution (50 parts Reagent A and 1 part Reagent B) to each tube and vortex. Incubate 30 minutes at 37°C.
8. Cool samples to RT and measure absorbance at 562 nm.
9. Plot a standard curve based on absorbance of the protein standards and determine the protein concentration of each unknown sample according to the BCA Protein Assay Kit instructions.

C. Protocol for Micro BCA Protein Assay

1. Pipette 500 µl of each standard (including a blank) and sample into 1.5 ml microcentrifuge tubes in triplicate.
2. Add 1 ml of cold acetone (-20°C) to each tube.
3. Vortex and incubate 30 minutes at -20°C.
4. Centrifuge 10 minutes at maximum speed in a microcentrifuge.
5. Pour off the supernatants and allow the acetone to evaporate from the tubes at RT for 30 minutes.

   Note: If two precipitations are necessary to remove the interfering substance, repeat Steps 2-5 before proceeding.
6. Add 500 µl of ultrapure water to the protein pellets and vortex.
7. Add 500 µl of Micro BCA Working Solution (50 parts Reagent A: 48 parts Reagent B: 2 parts Reagent C) to each tube and vortex. Incubate 60 minutes at 60°C.
8. Cool samples to RT and measure absorbance at 562 nm.
9. Plot a standard curve based on absorbance of the protein standards and determine the protein concentration of each unknown sample according to the Micro BCA Protein Assay Kit instructions.

Related Thermo Scientific Products

23215	**Compat-Able Protein Assay Preparation Reagent Set**, sufficient reagents to pre-treat 500 samples to remove interfering substances before total protein quantitation
23209	**Bovine Serum Albumin Standard Ampules, 2 mg/ml**, 10 × 1 ml ampules containing bovine serum albumin (BSA) at a concentration of 2.0 mg/ml in 0.9% saline and 0.05% sodium azide.
23208	**Bovine Serum Albumin Standard Pre-Diluted Set**, 7 × 3.5 ml aliquots in the range of 125-2000 µg/ml
23212	**Bovine Gamma Globulin Standard Ampules, 2 mg/ml,** 10 × 1 ml
23213	**Bovine Gamma Globulin Standard Pre-Diluted Set**, 7 × 3.5 ml aliquots in the range of 125-2000 µg/ml
23250	**Pierce BCA Protein Assay Kit – Reducing Agent Compatible**
23252	**Pierce Microplate BCA Protein Assay Kit – Reducing Agent Compatible**
23225, 23227	**Pierce BCA Protein Assay Kits**, working range 20-2000 µg/ml
23235	**Pierce Micro BCA Protein Assay Kit**, working range of 0.5-20 µg/ml
23236	**Pierce Coomassie Plus (Bradford) Protein Assay Kit**, working range of 1-1500 µg/ml

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.

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